AD_____

Award Number: DAMD17-02-1-0152

TITLE: Molecular Mechanisms of Prostate Cancer Progression

PRINCIPAL INVESTIGATOR: Shawn E. Holt, Ph.D.
Lynn W. Elmore, Ph.D.

CONTRACTING ORGANIZATION: Virginia Commonwealth University
Richmond, Virginia 23281-3039

REPORT DATE: January 2003

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	January 2003	Annual (19 Dec 01 - 18 Dec 02)

4. TITLE AND SUBTITLE
Molecular Mechanisms of Prostate Cancer Progression

5. FUNDING NUMBERS
DAMD17-02-1-0152

6. AUTHOR(S) :
Shawn E. Holt, Ph.D.
Lynn W. Elmore, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Virginia Commonwealth University
Richmond, Virginia 23281-3039

E-Mail: seholt@hsc.vcu.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

In studies to define the mechanisms involved in the progression of immortal, non-tumorigenic prostate cells to a tumorigenic state, we have found that molecular chaperones are elevated along with telomerase activity. Elevated chaperone function results in an increase in telomerase assembly. In order to determine the importance of the chaperone increase, we are investigating, both genetically and pharmacologically, whether ectopic chaperone expression results in transformation and if chaperones are targets for prostate cancer therapy. Individual chaperones have been over-expressed in non-tumorigenic prostate cells and are currently being tested for their ability to upregulate telomerase and other chaperone targets, as well as to influence tumorigenic growth (soft agar and nude mouse assays). In addition, treatment of malignant prostate cancer cell lines with radicicol, a specific hsp90 inhibitor, provides for a decline in telomerase activity and a decrease in overall telomere length, suggesting that chaperone inhibition provides an indirect means to block telomerase activation and is a plausible therapeutic option for prostate cancer treatment.

14. SUBJECT TERMS:	15. NUMBER OF PAGES
telomerase, chaperone, tumors, transformation, hsp90, radicicol	18
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Introduction

Telomerase is a cellular reverse transcriptase that is associated with over 90% of human prostate cancers and is composed of 2 integral components, an RNA template (hTR - human Telomerase RNA) and a catalytic polymerase (hTERT - human TElomerase Reverse Transcriptase) (Weinrich et al 1997). We have discovered that telomerase assembly is modulated by its interaction with the molecular chaperones in the hsp90 family, including hsp90, hsp70, p23 and hsp40 (Holt, et al., 1999). Using a highly relevant model for prostate cancer progression with non-tumorigenic, tumorigenic, and metastatic sublines with the same genetic background, we found that tumorigenic cells had 10-15-fold higher levels of telomerase activity than did the non-tumorigenic line and that there was no change in the expression profiles of the telomerase components, hTR and hTERT (Akalin, et al., 2001). We also found that the molecular chaperone family of proteins were upregulated as cell progressed to a more tumorigenic state, suggesting that the upregulation of telomerase activity is due to elevated assembly of the enzyme, rather than an increase of individual telomerase components. In fact, when purified chaperones are incubated with an extract from the non-tumorigenic line, telomerase is elevated, suggesting that this cell line harbors a significant amount of unfolded or improperly folded telomerase.

Body

Normal human prostate epithelial cells were immortalized by expression with the SV40 large T antigen oncogene, and an immortal, non-tumorigenic, telomerase-positive cell line was selected, P69. When injected into nude mice, no tumors formed within the standard 8-12 weeks, but if left in the animal for 6 months, 2 palpable sporadic tumors from a total of 19 mice formed after in vivo selection (Bae et al., 1994). Both lines, M2205 and M2182, were propagated in culture and reinjected into mice and were found to be tumorigenic. After undergoing another round of selection, the metastatic subline, M12, was generated. As our model for prostate cancer, the P69-M12 progression scheme provides an excellent system from a defined genetic background to study the molecular and cellular changes that occur during prostate cancer progression. Having found elevated telomerase levels in the more advanced prostate cancer lines and tumor samples (Akalin et al., 2001), our data indicates that this change in activity is due to an increase chaperone-mediated telomerase assembly rather than expression of the hTERT and hTR core components of the telomerase holoenzyme. As such, our goals are to determine if chaperones are the cause of the transformation event during prostate cancer progression and to show that chaperones are likely targets for anti-telomerase therapy in advanced prostate cancer.

Objective #1: Define the role of chaperones and telomerase activation during prostate cancer progression.

This first aim is designed to determine if the elevated chaperone expression levels can cause transformation in the non-tumorigenic P69 cells. We have designed and made retroviral constructs for chaperone-related genes and hTERT (Figure 1). Each construct can be utilized and stably selected into cells either alone or in combination with any of the other constructs. In addition, although we have had trouble subcloning the hsp70 cDNA into a retroviral construct, we will continue with this cloning, but because it is already in a transfectable plasmid with a CMV promoter, we will be able to express this in cells although at a lower transfection efficiency than retroviral infection.

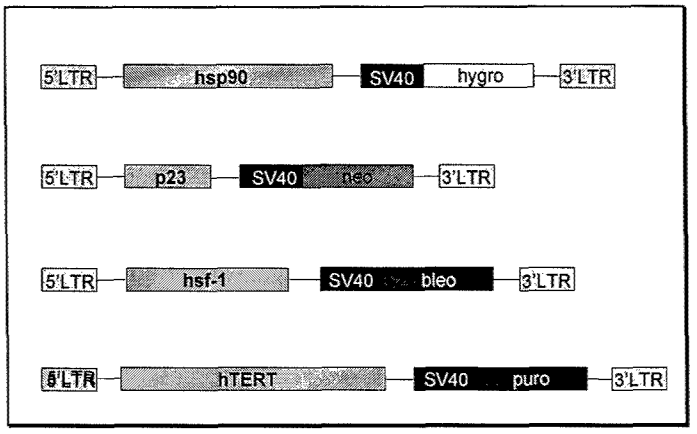

Figure 1. Constructs used for stable expression of chaperone-related proteins and telomerase. Each of the cDNAs for hsp90, p23, hsf-1, and hTERT was cloned into the pBABE retroviral vector system for expression in P69 cells. Importantly, each has a different selectable marker, meaning that each gene can be expressed alone or together with any of the others. Constructs are transfected in the Phoenix A cells and the viral supernatants are harvested at 48h for infection into P69.

Each construct was transiently transfected into the amphotropic mouse cell line, Phoenix A, the supernatant was harvested at 48h post-transfection, and the P69 cell line was infected with each of the constructs. The appropriate selection was accomplished and compared to uninfected controls, followed by recovery of pooled clonal populations. Because one of our primary observations was that increased chaperones result in elevated telomerase activity, we tested each stable cell population for an increase in telomerase activity levels as a means to determine over-expression of functional chaperone protein. Figures 2 and 3 show the results of infections with hTERT and p23 as populations of cells (Figure 2) and hsp90, hsf-1, and p23 as clonal populations (Figure 3).

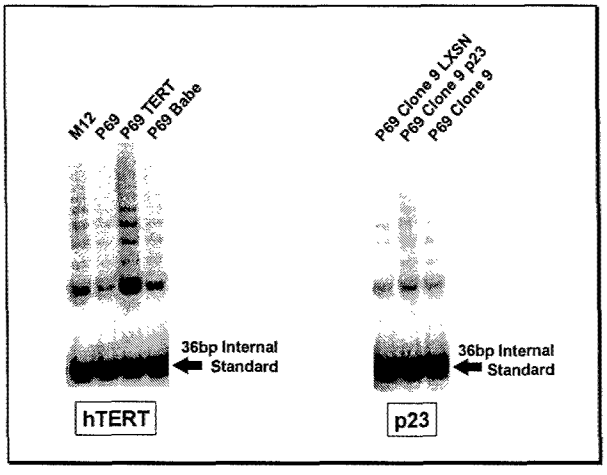

Figure 2. Expression of hTERT and p23 in the non-tumorigenic prostate cells results in increased telomerase activity. Selected populations of hTERT-infected cells and p23-infected cells were tested for telomerase activity using the TRAP (telomere repeat amplification protocol) assay as recommended by the manufacturer (Intergen, Gaithersburg, MD). The characteristic telomerase laddering effect is observed as well as the 36-bp internal standard that serves as a quantitative control.

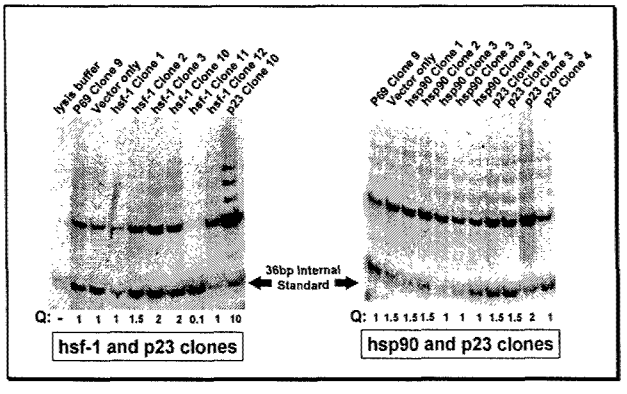

Figure 3. Clonal expansion of chaperone-infected P69 cells shows a more convincing increase in telomerase activity. Infected P69 cells were expanded for selection of single cell-derived clones and subsequently assayed for telomerase activity. Each lane was quantitated ('Q') and the numeric value is below each lane. Quantitation is accomplished by taking the ratio of the telomerase ladder to that of the 36-bp standard, compared to vector-controls (set as 1). Some clonal variability is observed, especially with the p23 cells.

For the selected populations of cells, a substantial increase is observed in the hTERT cells, while only a modest increase in activity in the p23-infected P69 cells. Interestingly, the hTERT cells have an increase in activity without a net increase in chaperone levels (no change in tumorigenic potential), and because our data suggests that telomerase activity is elevated by increased chaperones, these data present a conundrum. So, if the increase in activity is due to elevated chaperon-mediated telomerase assembly, this would suggest 2 things: 1-there is plenty of unfolded telomerase in P69 cells, and 2-no increase in the amount of telomerase core components should allow for the increase in telomerase activity if folding is the mechanism. Because we observe this substantial increase with hTERT-infected cells, we are currently investigating the hypothesis that because endogenous hTERT is such a low abundant target (less than 50 copies of mRNA per cell), over-expression of hTERT provides for accumulation of the hTERT protein, making it a more abundant target for the hsp90 chaperone machinery. We expect that if hsp90 targets telomerase more so in the presence of over-expressed hTERT, then we should observe an increase in hsp90 associated with telomerase activity and a decrease in the association with other hsp90-associated targets. This is currently under investigation.

Because the p23 population showed a modest increase that was suggestive of increased chaperone function (similar to the hsf-1 and hsp90 populations, not shown), we obtained single-cell-derived clones for each of the infected populations. We find some clonal heterogeneity with the p23 clones, ranging from 2-fold to 10-fold increased activity, suggesting that p23 is perhaps the limiting factor in the telomerase assembly process in P69 cells. Interestingly, we observe little in the way of an increase in telomerase activity (hence, chaperone function) with hsf-1 and hsp90, suggesting a potential feedback inhibition of chaperone function in infected cells. Because hsp90 associates with hsf-1 in the cytoplasm, this prevents hsf-1 from becoming a trimer through a complex mechanism involving multiple levels of phosphorylation and dephosphorylation, and prevents hsf-1 from trafficking to the nucleus to complete its function as a heat shock transcription factor (Zou et al., 1998). Over-expression may not completely compensate for this feedback inhibition, and other measures will be required to obtain transcriptionally functional hsf-1. Similarly for hsp90, if hsp90 associates with hsf-1 and blocks its ability to transactivate the heat shock promoters, including the hsp90 promoter, exogenous expression of hsp90 may increase its ability to block hsf-1 function, which would reduce the expression of the endogenous hsp90. We are currently investigating both of these possibilities.

Objective #2: Determine the cellular and molecular consequences of targeted inhibition of chaperones and/or telomerase using pharmacological and genetic approaches in tumorigenic prostate cancer cells.

Because we observe an increase in chaperone expression and function in prostate cancer cell lines and primary prostate tumors (Akalin et al., 2001), our goal was to determine if chaperones, specifically hsp90, were targets for inhibition of telomerase activity and reversion of the tumorigenic phenotype to a less severe, non-tumorigenic state. Thus far, we have successfully employed only the pharmacological approach to blocking chaperones as the antisense approaches to genetically down-regulating chaperones have been less informative. Our initial results with geldanamycin and radicicol provided an impetus to continue this line of treatment

and to follow treated cells for many months. Initially, we found that geldanmycin was quite toxic and resulted in a decline in telomerase as well as in cell viability, an outcome that was undesirable for an indirect method of telomerase inhibition (data not shown). However, radicicol, which binds in the ATP-binding pocket of hsp90 and blocks its ability to associate with p23 and other chaperone targets, was capable of blocking telomerase activity in a time-dependent manner (Figure 4). Using a chronic continuous replenishment of radicicol in the M12 cells, we found that telomerase activity (hence, chaperone function) was reduced to nearly undetectable levels after 14 days, the timing of which differs from subsequent results with differing treatment schedules.

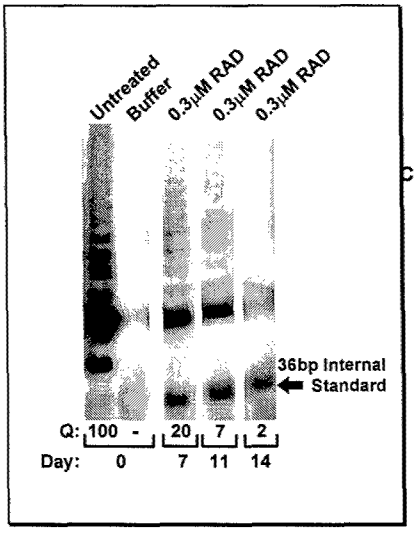

Figure 4. Inhibition of telomerase activity in the metastatic M12 prostate cancer subline after treatment with the hsp90 inhibitor, radicicol. Chronic treatment of M12 cells with radicicol (0.3 mM) for up to 2 weeks results in an inhibition of telomerase activity ('Q' denotes quantitation) without observable cell death. In addition, no change in growth rate was observed during the initial 2-week treatment schedule. As can be observed in later experiments, the timing of down-regulation is variable, but initially suggested a slow decrease in telomerase activity (Harvey et al., 2002).

Because of these promising initial results, cells were treated on differing regimens with radicicol for upwards of 3 months. Our initial pass with either 2-day or 4-day replenishment of drug provide strikingly novel results with an expected long-term outcome. Figures 5 and 6 show the telomerase activity, telomere length, and chaperone levels for both the 2-day schedule and the 4-day schedule. Interestingly, the 2-day treatments lead to a reduction in functional telomerase at the beginning of treatment, followed by a recovery of activity, which mimics the observed decline in chaperone levels (Figure 5). The 4-day treatments again show the transient telomerase decline in the absence of chaperone reduction or telomere shortening (Figure 6).

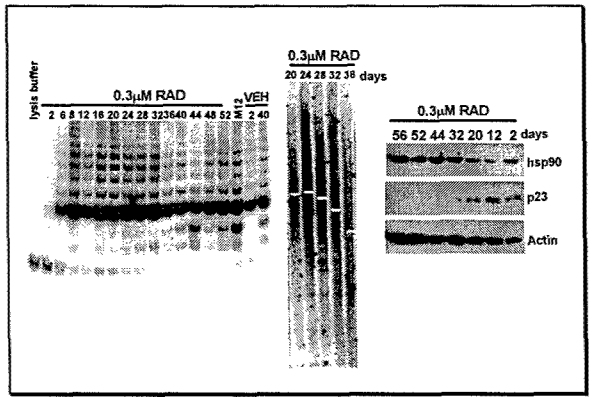

Figure 5. A 2-day schedule of radicicol replenishment provides for transient telomerase inhibition and chaperone loss, followed by long-term shortening of telomere lengths. Telomerase activity was measured by TRAP, telomere length (center) was assessed by TRF (terminal restriction fragment) as before (Bodnar et al., 1998), and chaperone levels by Western. We observe an initial decline in telomerase activity and chaperone levels followed by recovery and a delayed telomere shortening (median size indicated by white bars).

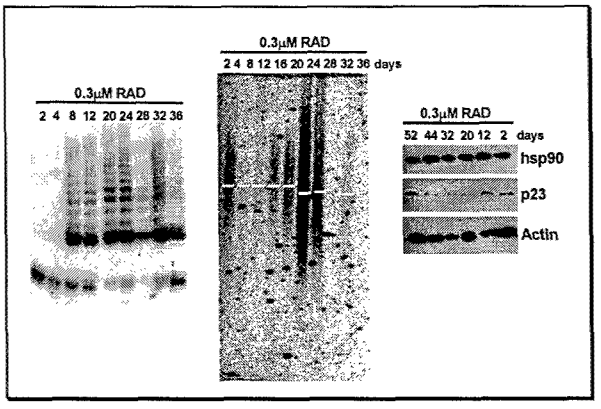

Figure 6. 4-day radicicol treatments show the transient telomerase inhibition without substantial changes in chaperone levels or telomere lengths. As in Figure 5, telomerase activity is reduced in a similar transient fashion, but no change in chaperone levels or telomere lengths is observed. White bars indicate the median telomere length (center panel).

Long-term treatments (out to 65 days) show differences in the biologic effect of the 2-day versus the 4-day treatments. With the 4-day, we observe a continued resistance to the 0.3 mM radicicol concentration, without changes in telomere length or telomerase activity after 60+ days (data not shown). However, the 2-day replenishment experiment provided a surprising, but somewhat expected result: sudden apoptosis at day 65. We are further characterizing the timing of this apoptotic effect and the molecular and cellular consequences of chaperone inhibition with respect to telomerase activity and telomere erosion. One mechanism that we are exploring is how telomeres can continue to shorten in the presence of active telomerase. Many possibilities exist, including telomere accessibility, subcellular localization, and post-translation modification. In addition, we have begun to utilize new compounds (e.g. novobiocin) to block chaperone function using a totally different mechanism of hsp90 inhbition, as well as innovative genetic approaches (siRNA) to block chaperone function and telomerase activity to determine if the cellular and molecular consequences observed for the pharmacologic inhibitors of molecular chaperones are specific for chaperones or telomerase or require an interaction of both.

Key Research Accomplishments

1-establishment of chaperone-expressing P69 cells that result in an increase in telomerase activity.

2-expression of molecular chaperones in non-tumorigenic cells results in a partial feedback inhibition of chaperone expression (will be further defined in terms of exogenous versus endogenous protein expression).

3-blocking chaperone function with radicicol results in transient telomerase inhibition, telomere erosion, and eventually cell death in tumorigenic cell lines, indicating that targeting chaperones in tumorigenic prostate cancer cells may be an appropriate therapy.

Recommended Changes to the Proposed Work Based on Additional Findings

There are 3 interesting issues that we will address related to each one of the specific aims. Within the first aim, over-expressed chaperones show a modest increase in expression levels and telomerase activity, suggesting a mechanism of feedback inhibition based on expressed levels of the chaperone proteins. By expressing hsf-1 phosphorylation mutants or blocking certain hsf-1 kinases in P69 cells, we may be able to eliminate the feedback inhibition by having

constituitively active hsf-1, which would result in the activation of other hsps and telomerase. We will also expand our work to include the investigation of this mechanism for chaperone suppression, as well as expand it to include ras-induced transformation of the P69 cells to determine if chaperone and telomerase expression profiles are similar to the in vivo selected sublines. In addition, we have expanded this work to include the hTERT overexpression and the mechanisms involved in how elevated hTERT would provide and increase in telomerase activity. In the second aim, we originally proposed utilizing antisense gene expression to genetically block chaperone expression in the tumorigenic lines. However, since the application was submitted, we have utilized small interfering RNAs to block to functions of telomerase and p53 in other cells lines and strains, and it is likely that we will utilize this siRNA technology to block the expression of molecular chaperones in order to revert the cells back to their non-tumorigenic state. In addition within this aim, we will utilize the pharmacological approach to determine the fate of the chaperone after drug treatment – i.e. half-life and protein degradation.

Reportable Outcomes

Manuscripts

Harvey,S.A., K.O.Jensen, L.W.Elmore, and **S.E.Holt**. 2002. Pharmacological approaches to defining the role of chaperones in aging and prostate cancer progression. Cell Stress and Chaperones 7:230-234.

Abstracts/Presentations

Holt,S.E., L.W.Elmore, A.Akalin, H.Forsythe, K.Jensen, S.Harvey, and J.L.Ware. New Discoveries in Prostate Cancer Biology and Treatment. AACR. Naples, FL. December 2001.

Harvey,S.A., A.Akalin, L.W.Elmore, D.A.Gewirtz, and **S.E.Holt**. American Cancer Society of Virginia Annual Meeting, Blacksburg, VA. April 2002.

Harvey,S.A., L.W.Elmore, and **S.E.Holt**. AACR: The role of telomeres and telomerase in cancer, San Francisco, CA. December 2002.

Invited Seminars

Holt,S.E. Bridges Program, Virginia State University, Petersburg, VA. November 2002.

Holt,S.E. Department of Pharmacology/Toxicology, MCV/VCU, Richmond, VA October 2002.

Holt,S.E. Department of Chemistry, MCV/VCU, Richmond, VA. September 2002.

Holt,S.E. Stem Cells on Land and at Sea. Mount Desert Island Biological Laboratory and Jackson Laboratory, Bar Harbor, ME. August 2002.

Holt,S.E. Department of Medicine, University of Colorado Health Science Center, Denver, CO. June 2002.

Holt,S.E. Department of Medical Microbiology and Immunology, Texas A&M Health Science Center, College Station, TX. April 2002.

Holt,S.E. Department of Biochemistry, University of Texas Health Center, Tyler, TX. April 2002.

Holt,S.E. Department of Physiology, University of Connecticut, Storrs, CT. February 2002.

Holt,S.E. Department of Pharmacology/Toxicology, Yale University, New Haven, CT. January 2002.

Development of Cell Lines

We have developed cell lines for telomerase over-expression in the non-tumorigenic prostate epithelial cell line, as well as individual P69 cell lines with oncogenic ras, hsp90, hsf-1, and p23 over-expression. Currently, we are in the process of making the corresponding tumorigenic cell lines knocking out hsp90, p23, telomerase, and hsf-1 using both antisense and siRNA approaches.

Funding Applied For

Department of Defense Breast Cancer Research Program, IDEA award, June 2002 – Declined

Conclusions

Having established numerous cell lines with over-expression of chaperone-related genes, telomerase, and oncogenic ras in the non-tumorigenic P69 cell line, we are clearly on pace to define the cellular consequences of ectopic expression of these proteins and their role in transformation. Over-expression of telomerase on its own is not sufficient to elicit transformation nor does it allow for elevation of the chaperone proteins. Increased chaperone expression has shown some promise in initial stable clones for activation of telomerase assembly, but additional genetic manipulations may be required (e.g. constituitively active hsf-1). Our initial goals for the pharmacologic inhibition of molecular chaperones and telomerase is not only interesting, but represents the first indirect method for invoking inhibition of telomerase function and subsequent telomere shortening in a prostate cancer cell model system. Inhibition of chaperone function in tumorigenic prostate cells may represent a novel mode of prostate cancer therapy that would be useful for patients with more severe disease and an increase in recurrence or metastasis.

References (**bold** indicates PI authored papers)

Akalin,A., L.W.Elmore H.L.Forsythe, B.A.Amaker, E.D.McCollum, P.S.Nelson, J.L.Ware, and S.E.Holt. 2001. A novel mechanism for chaperone-mediated telomerase regulation during prostate cancer progression. Cancer Research. 61:4791-4796.

Bae VL, Jackson-Cook CK, Brothman AR, Maygarden SJ, and Ware JL. 1994. Tumorigenicity of SV40 T antigen immortalized human prostate epithelial cells: association with decreased epidermal growth factor receptor (EGFR) expression. Int J Cancer **58**:721-729.

Bodnar AG, Ouellette M, Frolkis M, Holt SE, Chiu C-P, Morin GB, Harley CB, Shay JW, Lichtsteiner S, and Wright WE. 1998. Extension of life-span by introduction of telomerase into normal human cells. Science 279:349-352.

Harvey,S.A., K.O.Jensen, L.W.Elmore, and S.E.Holt. 2002. Pharmacological approaches to defining the role of chaperones in aging and prostate cancer progression. Cell Stress and Chaperones 7:230-234.

Holt,S.E., D.L.Aisner, J.Baur, V.M.Tesmer, M.Dy, M.Ouellette, D.O.Toft, J.B.Trager, G.B.Morin, W.E.Wright, J.W.Shay, and M.A.White. 1999. Functional requirement of p23 and hsp90 in telomerase complexes. Genes and Development 13:817-826

Weinrich,S.L., R.Pruzan, L.Ma, M.Ouellette, V.M.Tesmer, S.E.Holt, A.G.Bodnar, S.Lichtsteiner, N.W.Kim, J.B.Trager, R.D.Taylor, R.Carlos, W.H.Andrews, W.E.Wright, J.W.Shay, C.B.Harley, and G.B.Morin. 1997. Reconstitution of human telomerase with the template RNA hTR and the catalytic protein subunit hTRT. Nature Genetics 17: 498-502.

Zou,J., Y.Guo, T.Guettouche, D.F.Smith, and R.Voellmy. 1998. Repression of heat shock transcription factor hsf1 activation by hsp90 (hsp90 complex) that forms a stress-sensitive complex with hsf1. Cell **94**: 471-480.

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed on Form Page 2.
Photocopy this page or follow this format for each person.

NAME	POSITION TITLE
Shawn E. Holt, Ph.D.	Assistant Professor

EDUCATION/TRAINING (*Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.*)

INSTITUTION AND LOCATION	DEGREE (*if applicable*)	YEAR(s)	FIELD OF STUDY
The Colorado College, Colorado Springs, CO	B.A.	1985-1989	Biology
Texas A&M University, College Station, TX	Ph.D.	1989-1994	Genetics
The University of Texas Southwestern, Dallas, TX	Postdoc	1994-1998	Aging and Cancer

RESEARCH AND PROFESSIONAL EXPERIENCE:, INCLUDING GRANT SUPPORT. DO NOT EXCEED 3 PAGES.

1998-present	Assistant Professor, Department of Pathology and Department of Human Genetics, Virginia Commonwealth University/Medical College of Virginia, Richmond, VA
1998-present	Member, Massey Cancer Center, Virginia Commonwealth University/Medical College of Virginia, Richmond, VA
2002-present	Adjunct Faculty, Department of Pharmacology and Toxicology, MCV/VCU, Richmond, VA
2002-present	Member, Molecular Biology and Genetics Program, MCV/VCU, Richmond, VA

Awards and Honors:

2000-2003	The V Foundation Scholars Program, Cary, NC ($100,000 award)
1996-1998	NRSA Fellowship, National Institute on Aging, while at UT Southwestern, Dallas, TX
1994	Outstanding Presenter, Research Symposium, Texas A&M University, College Station, TX
1994	Outstanding Student Government Member, Texas A&M University, College Station, TX
1988-1989	Dean's List, The Colorado College, Colorado Springs, CO
1988	Most Dedicated Football Player, The Colorado College, Colorado Springs, CO
1987	Rookie of the Year, Baseball, The Colorado College, Colorado Springs, CO
1985-1987	Outstanding College Students of America

Publications (over the past 3 years, from a total of 38)

Morales CP, **S.E.Holt**, M.Ouellette, K.Kaur, Y.Yan, K.S.Wilson, M.A.White, W.E.Wright, and Shay JW. 1999. Absence of cancer-associated changes in human fibroblasts immortalized with telomerase. Nature Genetics **21**:55-58.

Holt,S.E., D.L.Aisner, J.Baur, V.M.Tesmer, M.Dy, M.Ouellette, D.O.Toft, J.B.Trager, G.B.Morin, W.E.Wright, J.W.Shay, and M.A.White. 1999. Functional requirement of p23 and hsp90 in telomerase complexes. Genes and Development **13**:817-826

Holt,S.E. and J.W.Shay. 1999. The role of telomerase in cellular proliferation and cancer. Journal of Cellular Physiology **180**:10-18.

Holt,S.E., V.V.Glinsky, A.B.Ivanova, and G.V.Glinsky. 1999. Resistance to apoptosis in human cells conferred by telomerase function and telomere length. Molecular Carcinogenesis **25**:241-248.

Tesmer,V.M., L.P.Ford, **S.E.Holt**, B.C.Frank, X.Yi, D.L.Aisner, M.Ouellette, J.W.Shay, and W.E.Wright. 1999. Two inactive fragments of the integral RNA cooperate to assemble active telomerase with the human protein catalytic subunit (hTERT) in vitro. Molecular and Cellular Biology **19**:6207-6216.

Savre-Train,I., L.S.Gollahon, and **S.E.Holt**. 2000. Clonal heterogeneity in telomerase activity and telomere length in tumor-derived cell lines. Proceedings of the Society for Experimental Biology & Medicine **223**:379-388.

Ouellette,M., M.Liao, B.Shea-Herbert, M.Johnson, **S.E.Holt**, H.S.Liss, J.W.Shay, and W.E.Wright. 2000. Subsenescent Telomere Lengths in Fibroblasts Immortalized by Limiting Amounts of Telomerase. Journal of Biological Chemistry **275**:10072-10076.

Elmore,L.W. and **S.E.Holt**. 2000. Telomerase and telomere stability: A new class of tumor suppressor? Molecular Carcinogenesis **28**:1-4.

Gollahon,L.S. and **S.E.Holt**. 2000. Alternative methods of extracting telomerase activity from human tumor and tissue samples. Cancer Letters **159**:141-149.

Forsythe,H.L. J.L.Jarvis, J.W.Turner, L.W.Elmore, and **S.E.Holt.** 2001. Stable association of hsp90 and p23 with human telomerase. J Biol. Chem. **276**:15571-15574.

Akalin,A., L.W.Elmore H.L.Forsythe, B.A.Amaker, E.D.McCollum, P.S.Nelson, J.L.Ware, and **S.E.Holt**. 2001. A novel mechanism for chaperone-mediated telomerase regulation during prostate cancer progression. Cancer Research. **61**:4791-4796.

Holt,S.E., E.J.Brown, and A.Zipursky. 2002. A study of telomerase levels in the leukemic cells of patients with the megakaryoblastic leukemias of Down syndrome. J Ped Hemat/Oncol. **24**:14-17.

Holt,S.E. 2002. Is Telomerase the Cure-All and End-All? Drug Discovery Today **7**:288.

Forsythe,H.L., K.O.Jensen, L.W.Elmore, and **S.E.Holt.** 2002. Ectopic expression of telomerase in human cells provides a growth advantage, extends proliferative life span, and rescues near senescent cells. International Journal of Oncology **20**:1137-1143.

McKinstry,R., L.Qiao, A.Yacoub, Y.Dai, R.Decker, **S.Holt**, M.P.Hagan, S.Grant, and P.Dent. 2002. nhibitors of MEK1/2 Interact with UCN-01 to Induce Apoptosis and Reduce Colony Formation in Mammary and Prostate Carcinoma Cells. Cancer Biology and Therapy **1**:243-253.

Harvey,S.A., K.O.Jensen, L.W.Elmore, and **S.E.Holt**. 2002. Pharmacological approaches to defining the role of chaperones in aging and prostate cancer progression. Cell Stress and Chaperones **7**:230-234.

Elmore,L.W., H.L.Forsythe, A.Ferreira-Gonzalez, C.T.Garrett, G.M.Clark, and **S.E.Holt.** 2002. Real-Time Quantitative Analysis of Telomerase Activity in Breast Tumor Specimens using a Highly Specific and Sensitive Fluorescent-based Assay. Diagnostic Molecular Pathology **11**: 177-185.

Elmore,L.W., K.C.Turner, L.S.Gollahon, M.R.Landon, C.K.Jackson-Cook, and **S.E.Holt**. 2002. Telomerase protects cancer-prone cells from chromosomal instability and spontaneous immortalization. Cancer Biology and Therapy **1**:395-401.

Elmore,L.W., C.W. Rehder, M.Di, P.A.McChesney, C.K.Jackson-Cook, D.A.Gewirtz, and **S.E.Holt**. 2002. Adriamycin-induced replicative senescence in tumor cells requires functional p53 and telomere dysfunction. J. Biol. Chem. **277**:35509-35515.

McChesney,P.A., L.W.Elmore, and **S.E.Holt**. 2002. Aging and oxidative damage: are telomeres the target? Comments on Theoretical Biology **7**: 295-313..

Wise,S.S., L.W.Elmore, P.G.Antonucci, **S.E.Holt**, B.H.Bryant, J.P.Wise. 2003. Establishing a human bronchial cell culture fibroblast model for lung carcinogens. In press, Cellular and Molecular Biochemistry.

Ossum,C.G., E.K.Hoffman, M.M.V, S.E.Holt, and N.C.Bols. 2003. Isolation and characterization of a novel fibroblast-like cell line from the rainbow trout (Oncohynchus mykiss) and a study of p38MAPK activation and induction of HSP70 in response to chemically-induced ischemia. Submitted.

McChesney,P.A., K.C.Turner, L.W.Elmore, C.K.Jackson-Cook, and **S.E.Holt**. 2003. TRF2 cooperates with telomerase to stabilize telomeres. In preparation.

Forsythe,R., L.W.Elmore, H.L.Forsythe, S.Nasim, and **S.E.Holt**. 2003. Association of chaperones and telomerase in prostate tumor samples. In preparation.

Book Chapters

1-Busbee,D., S.Miller, M.Schroeder, V.Srivastava, B.Guntapalli, E.Merriam, **S.Holt**, V.Wilson, and R.Hart. 1995. DNA polymerase α Function and Fidelity: Dietary restriction as it affects age-related enzyme changes. International Life Sciences Institute; Risk Assesment Symposium.

2-Morales,C.P. and **S.E.Holt**. 2000. Detection of Telomerase by *In Situ* Hybridization and by the PCR-based Telomerase Activity Assay (TRAP). Methods in Molecular Medicine, Molecular Pathology Protocols, Humana Press. pp. 43-58.

3-Elmore,L.W. and **S.E.Holt**. 2001. Cell Proliferation, Telomerase and Cancer. Advances in Cell Aging and Gerontology: Telomerase, Aging and Disease. Elsevier Science, Inc. pp. 89-102.

4-Elmore,L.W. and **S.E.Holt**. 2002. Diagnosis and Treatment of Human Disease Using Telomerase as a Novel Target. Cancer Drug Discovery and Development, Humana Press. In press.

Existing/Pending Support

Active

P.I.: Shawn E. Holt, Ph.D.
Title: The Role of Telomerase in Human Cancer Development
Agency: The V Foundation Scholars Program
Amount: $100,000 total
Duration: 7/1/00 -6/30/03

P.I.: Shawn E. Holt, Ph.D.
Title: Therapeutic Strategies for the Prevention and Treatment of Breast Cancer Using Telomerase
Agency: The Mary Kay Ash Charitable Foundation
Amount: $100,000 total
Duration: 7/1/00 -6/30/03

P.I.: Shawn E. Holt, Ph.D.
Title: Functional characterization of primary mammary cells with life span extension using ectopic telomerase expression
Agency: Department of Defense
Amount: $72,500 total
Duration: 8/1/01-8/31/03

P.I.: David A. Gewirtz, Ph.D. (Shawn E. Holt, Ph.D., Co-PI, 10% effort)
Title: Reciprocal regulation of senescence/apoptosis in response to adriamycin in the breast tumor cell
Agency: Department of Defense
Amount: $450,000 total
Duration: 7/1/01-6/30/04

P.I.: Shawn E. Holt, Ph.D.
Title: Mechanisms of Prostate Cancer Transformation
Agency: Department of Defense
Amount: $320,000 total
Duration: 12/18/01-12/17/04

Agency: NIEHS (ES03828)
Investigator: Shawn E. Holt, Ph.D. (pilot project funding)
Amount: $12,000 total
Duration: 6/1/02-9/15/03

P.I.: Colleen Jackson-Cook, Ph.D. (Shawn E. Holt, Ph.D., Co-PI, 10% effort)
Title: Aging and genomic changes: role of enviroment/genetics
Agency: NIH
Amount: $220,000 direct
Duration: 11/1/02-10/31/04

Pending Grants

none

Perspective

Cell Stress & Chaperones (2002) **7** (2), 230–234

Article no. csac. 2002.383

Pharmacological approaches to defining the role of chaperones in aging and prostate cancer progression

Sarah A. Harvey,[1] Keith O. Jensen,[2] Lynne W. Elmore,[3] and Shawn E. Holt[2,3,4]

[1]Department of Pharmacology and Toxicology, [2]Department of Human Genetics, [3]Department of Pathology, [4]Massey Cancer Center, Medical College of Virginia at Virginia Commonwealth University, 1101 East Marshall Street, Richmond, VA 23298-0662, USA

The underlying mechanisms of human aging are as yet not clearly defined partly because of the complexity and the intricate pathways involved in the process. There are clearly a number of environmental and genetic challenges that contribute to a shortened cellular life span, but investigations into their interrelationships have received less attention. A diminished stress response and decreased function of the molecular chaperones hsp90 and hsp70 (Gutsmann-Conrad et al 1998) appears to contribute to replicative senescence (Lee et al 1996), which can be overcome by chronic stress and elevated chaperone levels. There is accumulating evidence to suggest that both a mild heat shock of human fibroblast cultures (Rattan 1998) and forced expression of hsp70 in invertebrates (Wheeler et al 1995) lead to enhanced tolerance to environmental stress (hormesis) resulting in increased longevity. Additional aging mechanisms that have been proposed implicate oxidative damage together with accelerated telomere erosion as causative agents of premature senescence. For example, by culturing cells in low oxygen (hypoxic conditions) or in the presence of reactive oxygen species traps, primary human cells exhibit a reduced rate of telomere shortening and an extended life span (von Zglinicki et al 2000), suggesting that culturing cells in "normoxia" (standard 20%) leads to increased telomere erosion and decreased life span. It is clear that a number of factors play important roles in the onset of senescence, from oxidative damage to a depressed stress response to telomere erosion. Consistent with these hypotheses and data suggesting that cancer is associated with an increase in chaperone function, a decline in the stress response during senescence may be critical for this proliferative block, eventually resulting in the prevention of immortalization and progression to cancer. We discuss here telomeres and telomerase as they relate to chaperones, stress response, therapy, aging, and ultimately cancer as a disease of aging.

Correspondence to: Shawn E. Holt, Tel: 804 827-0458; Fax: 804 828-5598; E-mail: seholt@hsc.vcu.edu.

Received 12 March 2002; Accepted 26 March 2002.

CONNECTING TELOMERES AND THE STRESS RESPONSE DURING SENESCENCE

Normal human cells are unable to proliferate indefinitely, whether in the human body or when explanted into tissue culture. Clearly, utilization of in vivo models most accurately addresses organismal aging; yet, in vitro systems provide an appropriate model to study certain pathways related to the cellular senescence program. The proliferative checkpoint to continuous cell division has been defined as replicative senescence (Hayflick and Moorhead 1961). The senescence process appears to correspond directly with the aging of the organism as a whole, given that cells cultured from a child divide more number of times than cells from an aged adult. In addition, as human cells approach senescence, they show a severe loss of responsiveness to environmental stress. Heat shock followed by recovery has been shown to provide a sustained stress response and an extension of cellular life span (Rattan 1998), presumably via a cytoprotective mechanism known as hormesis. Hormesis is defined as the adaptation or tolerance of cells or organisms to repeated mild stress in order to survive certain types of lethal environmental

challenges. Additional data suggest that chaperone introduction into invertebrate animals results in an extension of life span during organismal aging (Wheeler et al 1995). Recent results in Drosophila indicate a protective role for chaperone function in the prevention of neurodegenerative disorders, such as Parkinson's disease (Auluck et al 2002), further suggesting the decreased function of chaperones during aging.

Our current understanding directly relates the onset of senescence with the shortening of telomere repeats, specialized structures at the ends of the chromosomes. Each time a cell divides telomeric deoxyribonucleic acid (DNA) is lost because of the inability of conventional DNA polymerases to replicate to the end of the linear chromosome (Harley et al 1990). Amazingly, the introduction of the enzyme responsible for telomere maintenance, telomerase, blocks telomere erosion in normal cells, ultimately preventing cellular senescence (Bodnar et al 1998; Vaziri and Benchimol 1998) without signs of cancer-associated changes (Jiang et al 1999; Morales et al 1999). Thus, although there are a variety of theories and hypotheses on human aging, including increased oxidative damage or depressed response to environmental stress (or both), these data provided the first direct experimental evidence that telomere shortening is a primary cause of cellular senescence. Consistent with these observations are studies in telomerase knockout mice that show telomere shortening is directly related to organismal aging (Rudoph et al 1999), indicating that in vitro human models are an appropriate substitute for defining mechanisms related to aging in humans. It seems reasonable to suggest that human cells exposed to a sustained stress response or increased expression of chaperones would exhibit slower rates of telomere shortening compared with unstressed cells. However, maintaining a stress response may instead reduce the threshold level where telomeres are recognized as damaged DNA. Alternatively, because it has been recently shown that the shortest telomere is responsible for senescence and not the overall telomere lengths (Hemann et al 2001), it is possible that in cells with a sustained stress response, shortening of the shortest telomere may not occur at the same rate as untreated cells, leading to life span extension.

The interplay between stress, telomeres, telomerase, aging, and diseases of aging (namely, cancer) has yet to be experimentally clarified. The telomerase enzyme is minimally composed of a protein component, human telomerase reverse transcriptase (hTERT), the catalytic subunit (Meyerson et al 1997; Nakamura et al 1997), and a template ribonucleic acid (RNA), human telomerase RNA component (hTR), which is responsible for recognizing the 3′ overhang of the chromosome ends to template telomere addition (Feng et al 1995). The fact that telomerase migrates as a large molecular weight complex in glycerol gradients suggests that a number of proteins associate with the core components of telomerase (Bednenko et al 1997). Although there are a number of identified proteins that associate with telomerase, the chaperone proteins hsp90, hsp70, and p23 were the first shown to functionally associate with telomerase and to be required for telomerase assembly in vivo and in vitro (Holt et al 1999). Proteins hsp90 and p23 appear to remain associated with active telomerase to aid conformational changes that may take place as the enzyme translocates along the telomere to catalyze the addition of the next telomeric repeat. Data indicate that hsp70 interacts with hTERT, but unlike the stable association of hsp90 and p23, it dissociates upon proper conformation and activation of telomerase (Forsythe et al 2001). The minimal components required to assemble the human telomerase were identified as hTERT, hTR, hsp90, p23, hsp70, hsp40, and the heat shock organizing protein, HOP/p60 (Holt et al 1999). To date, the roles of the hsp40 and HOP proteins in telomerase assembly and regulation have not been determined but may be related to the kinetics of the assembly process. Given that telomere erosion, telomerase activity, and chaperones appear functionally interrelated, it is reasonable to assume that there may be a link between DNA damage, stress response, and telomere shortening related to the onset of replicative senescence or the immortalization-transformation process (or both).

PROSTATE CANCER THERAPY: THE UNIQUE TARGETING OF CHAPERONE FUNCTION

As a condition of the aging process, cancer may have a unique place as a disease where chaperone proteins play an integral role in therapeutic intervention. We have begun to dissect out the mechanisms involved in prostate cancer progression using an appropriate model system within a similar genetic background. Nonneoplastic prostate epithelial cells obtained from a radical prostatectomy were immortalized using SV40 large T antigen and shown to be nontumorigenic in the athymic nude mouse under standard conditions. However, after a latent 6-month incubation of the immortalized P69SV40T cells in the athymic mouse, 2 out of 18 mice developed sporadic tumors during an in vivo selection process, followed by in vitro culture and subsequent subcutaneous reinjection into mice to produce metastatic sublines. Therefore, the model system consists of cell lines from nontumorigenic (P69), tumorigenic (M2182), and metastatic (M12) cells of the same genetic lineage and cytogenetic aberrations, consistent with authentic human prostate cancer (Bae et al 1994, 1998).

Our studies with this model indicated that there is a dramatic increase in telomerase (~15-fold) from the nontumorigenic cells (P69) to the tumorigenic (M2182) and

metastatic (M12) cells, again supporting the critical role of telomerase in cancer progression (Akalin et al 2001). We also detect a substantial increase in chaperones and chaperone-related proteins in tumorigenic and metastatic cells, which directly correlates with the observed elevation in telomerase levels. These increases were found to be directly related: the change in telomerase activity levels was almost exclusively caused by increased chaperone-mediated assembly rather than elevated expression of the telomerase components. This is the first known model system to suggest the importance of chaperone proteins and telomerase in the transformation process in prostate cancer and identifies a novel, potentially important pathway for chaperone-mediated telomerase assembly during cancer progression. To determine if the increase in chaperone proteins is biologically significant, we have immunohistochemically analyzed nearly 75 primary prostate biopsy cases and found that chaperone up-regulation is consistently associated with tumorigenic conversion to prostate carcinoma (in preparation). Our data indicate that the levels of hsp90 and p23 expression are consistently (greater than 95% of the cases) higher in prostatic intraepithelial neoplasia and prostate carcinoma, relative to benign prostatic hyperplasia (in preparation). Because we find a dramatic increase in chaperones and chaperone-related proteins during tumorigenic conversion both in vitro and in vivo, these proteins have become novel targets for chemotherapeutic prostate cancer intervention.

Accumulating evidence suggests that certain hsp90 inhibitory compounds block the chaperone-mediated assembly of functional enzyme complexes. Of these drugs, radicicol and geldanamycin and its derivatives have shown the most promise. Radicicol is an antifungal antibiotic that was shown to inhibit transformation by oncogenes v-src, ras, raf, and mos (Kwon et al 1992; Zhao et al 1995). Isolated from the fungus *Monosporium bonorden*, radicicol is the prototype of a second class of hsp90 inhibitors that is unrelated structurally to the first class of inhibitors, the benzoquinone antibiotics (eg, geldanamycin and herbimycin A) (Schulte et al 1998). All of these hsp90 inhibitors have been shown to bind to the N-terminal adenosine triphosphate (ATP)–adenosine diphosphate (ADP) binding site of hsp90, where they compete for ATP binding and trap the molecule in the ADP-bound conformation, thereby inhibiting hsp90 function (Grenert et al 1997; Roe et al 1999; Schulte et al 1999). Radicicol has been shown to bind to all the hsp90 family members, including hsp90 α and β, Grp94 (the hsp90 homolog in the endoplasmic reticulum), and the newly discovered Trap-1 (hsp75), although it has a greater affinity for hsp90 (Schulte et al 1999). Recent studies suggest a limited clinical usefulness of these drugs because of their hepatotoxicity and narrow therapeutic index (Supko et al 1995); yet, newer synthetic analogs, like the geldanamycin analog

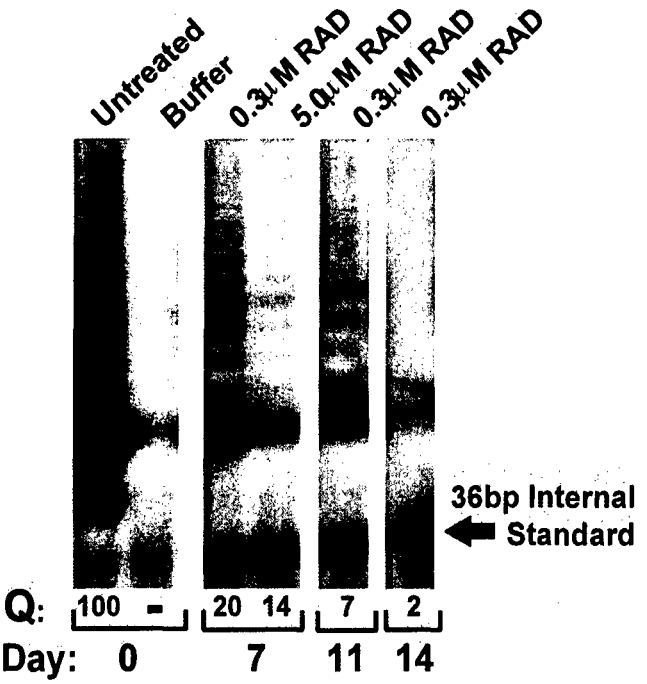

Fig. 1. Specific inhibition of the chaperone-mediated telomerase assembly with chronic radicicol treatment of the prostate cancer cells. The tumorigenic, metastatic prostate cancer cell line, M12, was treated with 0.3 μM and 5.0 μM radicicol (RAD) for the indicated days and tested for the inhibition of telomerase activity using the Telomeric Repeat Amplification Protocol (TRAP) assay (Kim et al 1994). A characteristic 6-bp telomerase-specific laddering is observed in the presence of telomerase activity (eg, lane 1), whereas the lysis buffer only (Buffer) serves as an appropriate negative control. The 36-bp internal standard serves as a useful internal control for the normalization of sample to sample variation, as well as for semiquantitative purposes. Quantitation (Q) is accomplished by obtaining the ratio of the telomerase ladder to the internal control, with untreated samples normalized to 100%. Although growth rates of the M12 cells were not affected by 0.3 μM radicicol, a gradual and specific inhibition of the telomerase activity was observed after long-term treatment. A 5.0-μM treatment was cytotoxic and caused substantial cell death (not shown) with an obvious decline in telomerase activity.

17-(allylamino)-17-demethoxygeldanamycin (17AAG), are proving to have a better therapeutic index (Burger et al 1998). In addition, other drugs (novobiocin and related coumarins) that are reported to target hsp90 are now being identified (Marcu et al 2000a, 2000b) and are already established as well-tolerated compounds.

Our initial studies using the prostate cancer progression model suggest that we can successfully block telomerase assembly using chronic treatments of the hsp90 inhibitor radicicol to target telomerase activity therapeutically. At subtoxic concentrations that do not significantly affect cell growth rates, we are able to dramatically reduce the telomerase activity (Fig 1), which is an indicator of chaperone inhibition. Our aim is to further extend these encouraging results in terms of telomere shortening and induction of senescence or cell death and to identify additional compounds that will serve as prostate cancer

inhibitors. We believe that we can target the excess or elevated functional chaperones observed in the tumorigenic cells to deplete telomerase activity, triggering telomere shortening and reprogramming of replicative senescence or induction of cell death. This novel treatment strategy may prove invaluable in the treatment of cancers, including prostate and breast. Although such a novel therapy for prostate cancer is warranted, it would likely be an adjuvant treatment, with general tumor resection as the first line of defense. Treatments for chaperone inhibition as a means to block telomerase assembly would be useful to aid in preventing the repopulation of the more aggressive cancerous cells left behind after the primary treatment (ie, tumor resection). It is widely believed that the primary tumor in many cases is not fatal; yet, the recurrence of a secondary tumor with invasive and metastatic capabilities results in increased mortality rates. On the basis of our recent data, specific inhibition of telomerase or telomerase-associated proteins as an adjuvant therapy for preventing recurrence of disease, especially in prostate cancer, may be a realistic objective.

Interestingly, recent studies have suggested that geldanamycin-mediated inhibition can be directed at specific targets. For example, geldanamycin-testosterone hybrids have been shown to have selective activity toward cancer cells that express the androgen receptor (Kuduk et al 2000), whereas geldanamycin dimers (ie, GMD-4c) target HER-kinase in HER-kinase overexpressing cancer cells (Zheng et al 2000). These synthetic molecules may prove to be useful in identifying novel approaches for the inhibition of specific hsp90-protein interactions without the toxic side effects associated with targeting all hsp90 targets in all cell types. As more compounds are identified as hsp90 inhibitors, the discovery of more specific and less toxic chemotherapeutic drugs that are well tolerated in humans will likely follow.

REFERENCES

Akalin A, Elmore LW, Forsythe HL, et al. 2001. A novel mechanism for chaperone-mediated telomerase regulation during prostate cancer progression. *Cancer Res* 61: 4791–4796.

Auluck PK, Chan HYE, Trojanowski JQ, Lee VMY, Bonini NM. 2002. Chaperone suppression of α-synuclein toxicity in a *Drosophila* model for Parkinson's disease. *Science* 295: 865–868.

Bae VL, Jackson-Cook CK, Brothman AR, et al. 1994. Tumorigenicity of SV40 T antigen immortalized human prostate epithelial cells: association with decreased epidermal growth factor receptor (EGFR) expression. *Int J Cancer* 58: 721–729.

Bae VL, Jackson-Cook CK, Maygarden SJ, et al. 1998. Metastatic sublines of an SV40 large T antigen immortalized human prostate epithelial cell line. *Prostate* 34: 275–282.

Bednenko J, Melek M, Greene EC, et al. 1997. Developmentally regulated initiation of DNA synthesis by telomerase: evidence for factor-assisted de novo telomere formation. *EMBO J* 16: 2507–2518.

Bodnar AG, Ouellette M, Frolkis M, et al. 1998. Extension of lifespan by introduction of telomerase into normal human cells. *Science* 279: 349–352.

Burger AM, Fiebig HH, Newman DJ, et al. 1998. Antitumor activity of 17-(allylamino)-17-demethoxygeldanamycin (NCS-330507) in melanoma xenographs is associated with decline in Hsp90 protein expression [abstract]. *Ann Oncol* 9: 504.

Feng J, Funk WD, Wang SS, et al. 1995. The RNA component of human telomerase. *Science* 269: 1236–1241.

Forsythe HL, Jarvis JL, Turner JW, et al. 2001. Stable association of hsp90 and p23, but not hsp70, with active human telomerase. *J Biol Chem* 276: 15571–15574.

Grenert JP, Sullivan WP, Fadden P, et al. 1997. The amino-terminal domain of heat shock protein 90 (hsp90) that binds geldanamycin is an ATP/ADP switch domain that regulates hsp90 conformation. *J Biol Chem* 272: 23843–23850.

Gutsmann-Conrad A, Heydari AR, You S, et al. 1998. The expression of heat shock protein 70 decreases with cellular senescence in vitro and in cells derived from young and old human subjects. *Exp Cell Res* 241: 404–413.

Harley CB, Futcher AB, Greider CW. 1990. Telomeres shorten during ageing of human fibroblasts. *Nature* 345: 458–460.

Hayflick L, Moorhead PS. 1961. The serial cultivation of human diploid cell strains. *Exp Cell Res* 25: 585–621.

Hemann MT, Strong MA, Hao LY, et al. 2001. The shortest telomere, not average telomere length, is critical for cell viability and chromosome stability. *Cell* 107: 67–77.

Holt SE, Aisner DL, Baur J, et al. 1999. Functional requirement of p23 and hsp90 in telomerase complexes. *Genes Dev* 13: 817–826.

Jiang XR, Jimenez G, Chang E, et al. 1999. Telomerase expression in human somatic cells does not induce changes associated with a transformed phenotype. *Nat Genet* 21: 111–114.

Kim NW, Piatyszek MA, Prowse KR, et al. 1994. Specific association of human telomerase activity with immortal cells and cancer. *Science* 266: 2011–2015.

Kuduk SD, Harris TC, Zheng FF, et al. 2000. Synthesis and evaluation of geldanamycin-testosterone hybrids. *Bioorg Med Chem Lett* 10: 1303–1306.

Kwon HJ, Yoshida M, Abe K, et al. 1992. Radicicol, an agent inducing the reversal of transformed phenotypes of src-transformed fibroblasts. *Biosci Biotechnol Biochem* 56: 538–539.

Lee YK, Manalo D, Liu AY. 1996. Heat shock response, heat shock transcription factor and cell aging. *Biol Signals* 5: 180–191.

Marcu MG, Chadli A, Bouhouche I, et al. 2000a. The heat shock protein 90 antagonist novobiocin interacts with a previously unrecognized ATP-binding domain in the carboxyl terminus of the chaperone. *J Biol Chem* 275: 37181–37186.

Marcu MG, Schulte TW, Neckers L. 2000b. Novobiocin and related coumarins and depletion of heat shock protein 90-dependent signaling proteins. *J Natl Cancer Inst* 92: 242–248.

Meyerson M, Counter CM, Eaton EN, et al. 1997. hEST2, the putative human telomerase catalytic subunit gene, is up-regulated in tumor cells and during immortalization. *Cell* 90: 785–795.

Morales CP, Holt SE, Ouellette M, et al. 1999. Absence of cancer-associated changes in human fibroblasts immortalized with telomerase. *Nat Genet* 21: 115–118.

Nakamura TM, Morin GB, Chapman KB, et al. 1997. Telomerase catalytic subunit homologs from fission yeast and humans. *Science* 277: 955–959.

Rattan SIS. 1998. Repeated mild heat shock delays ageing in cultured human skin fibroblasts. *Biochem Mol Biol Int* 45: 753–759.

Roe SM, Prodromou C, O'Brien R, et al. 1999. Structural basis for inhibition of the hsp90 molecular chaperone by the antitumor antibiotics radicicol and geldanamycin. *J Med Chem* 42: 260–266.

Rudoph KL, Chang S, Lee H-W, et al. 1999. Longevity, stress re-

sponse, and cancer in aging telomerase-deficient mice. *Cell* 96: 701–712.

Schulte TW, Akinaga S, Murakata T, et al. 1999. Interaction of radicicol with members of the heat shock protein 90 family of molecular chaperones. *Mol Endocrinol* 13: 1435–1448.

Schulte TW, Akinaga S, Soga S, et al. 1998. Antibiotic radicicol binds to the N-terminal domain of hsp90 and shares important biologic activities with geldanamycin. *Cell Stress Chaperones* 3: 100–108.

Supko JG, Hickman RL, Grever MR, et al. 1995. Preclinical pharmacologic evaluation of geldanamycin as an antitumor agent. *Cancer Chemother Pharmacol* 36: 305–315.

Vaziri H, Benchimol S. 1998. Reconstitution of telomerase activity in normal human cells leads to elongation of telomeres and extended replicative life span. *Curr Biol* 8: 279–282.

von Zglinicki T, Pilger R, Sitte N. 2000. Accumulation of single-strand breaks is the major cause of telomere shortening in human fibroblasts. *Free Radic Biol Med* 28: 64–74.

Wheeler JC, Bieschke ET, Tower J. 1995. Muscle-specific expression of Drosophila hsp70 in response to aging and oxidative stress. *Proc Natl Acad Sci U S A* 92: 10408–10412.

Zhao JF, Nakano H, Sharma S. 1995. Suppression of RAS and MOS transformation by radicicol. *Oncogene* 11: 161–173.

Zheng FF, Kuduk SD, Chiosis G, et al. 2000. Identification of a geldanamycin dimer that induces the selective degradation of HER-family tyrosine kinases. *Cancer Res* 60: 2090–2094.